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TITLE: Structural and Functional Analysis of CA125: Potential for Early Diagnosis and Understanding the Immune Evasion Strategies of Epithelial Ovarian Tumors

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14. ABSTRACT: The proposed studies will define the structure and the biological function of the epithelial ovarian tumor marker MUC16 (CA125). The knowledge obtained can be utilized to develop better diagnostic tests for early detection of ovarian cancer and also to understand the pathogenesis of this disease. Due to delays in procuring the appropriate approvals from the Institutional Review Boards we have not been able to initiate the proposed studies. Research on MUC16 in the PI's laboratory has continued, however, through the use of start-up funds provided by the University of Wisconsin-Madison. This research has led to a very in-depth understanding of the MUC16 molecule and its physiological relevance. The impact of this understanding on the proposed studies will be significant. Here, we briefly discuss the highlights of this research effort.					
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INTRODUCTION: The major molecular marker for epithelial ovarian cancer is the antigen CA125. It is now known that CA125 is repeating peptide epitope that is expressed on the very large molecular weight mucin MUC16. The major goal of this project was to further understand the structure and biology of CA125 (hereafter referred to as MUC16) in order to enhance the capability of the serum CA125 test to detect epithelial ovarian cancer and also to understand the role of MUC16 in the pathogenesis of this malignancy. For this purpose we proposed to carefully analyze the oligosaccharides attached to MUC16 that was isolated from ovarian tumor cells *in vitro*, from the peritoneal fluid of ovarian cancer patients and from the amniotic fluid derived from pregnant women. Our hypothesis was that this analysis would help us distinguish between the different isoforms of the mucin. In the second and the third specific aims we proposed to study the role of MUC16 in inhibiting the innate (Natural killer; NK) cell and adaptive (T-cell) responses. The three specific aims of the proposal are listed below.

Specific Aim 1- Perform glycomic analysis of CA125 derived from cultured cells, ascites of EOC patients and human amniotic fluid.

Specific Aim 2- Determine if cell surface associated CA125 can protect OVCAR-3 cells from NK cell mediated cytolytic responses.

Specific Aim 3- Determine if CA125 can modulate the function of T cells.

BODY: At the very outset we should state that we have not yet used any funds provided through this Department of Defense (DOD) grant. This was primarily because of the delay in acquiring the requisite approvals from the Institutional Review Boards of the PI's institution. The final IRB approvals for this study are expected in the first week of August, 2006. Only after that we will start utilizing the funds provided through this grant.

We would like to include here data that we have accumulated to date, using funds from sources other than the DOD, that are going to have a significant positive impact on our future studies that will be conducted as proposed in this grant. These new advances in our understanding of the structure and function of MUC16 are listed below.

Understanding the structure of MUC16. In order to perform the glycomic analysis of MUC16 from different sources it is imperative that we purify this mucin from complex biological fluids. We initially used the spent media from the ovarian tumor cell line OVCAR-3 to standardize purification protocols. We have now established that purification of the MUC16 can be achieved by serial chromatography on Sepharose CL-4B column followed by ion-exchange separation on a Q-Sepharose medium. Through all of these studies we have determined that the most highly purified MUC16 sample has a specific activity of 2.5×10^6 U of CA125 per milligram of total protein. Thus in all of our future experiments we will only utilize MUC16 samples that have this specific activity before conducting the glycomic analysis. This is an important step as it clearly sets the standard for purity that we must achieve when we initiate our analysis of MUC16 from the ascites and amniotic fluid samples.

It should also be noted that we have now set up collaboration with Dr. Lloyd Smith (Department of Chemistry, University of Wisconsin-Madison) to conduct proteomic analysis of the MUC16 samples. This collaboration with a local research group provides us with a rapid way to obtain very sensitive mass spectrometric data on the MUC16 samples that we isolate from ascites and the amniotic fluid samples. This data will provide further clarification of the purity of the isolated MUC16 samples. Once the purity has been confirmed the samples will be shipped to Dr. Anne Dell's laboratories at the Imperial College, London where the glycomic analysis of MUC16 will be carried out as proposed in the grant.

Another exciting development is that we have obtained approximately 30 different anti-MUC16 monoclonal antibodies through our collaboration with Neoclone technologies, a biotechnology company based in Madison Wisconsin. The access to these antibodies will help us in our quest to isolate very pure MUC16 samples in the future.

The high molecular weight of MUC16 prevents successful analysis of its size by using conventional polyacrylamide gel electrophoresis. We have now developed protocols to successfully analyze MUC16 by agarose gel (Fig 1). Using this technique we can demonstrate the differences in the mobilities of MUC16 that is shed from the cell (soluble MUC16) and that which is present on the ovarian tumor cells.

All of these new developments give us great confidence in successfully completing the studies outlined in the first specific aim of the grant.

Role of MUC16 in blocking NK cell function. In the preliminary results provided with the grant application we demonstrated that soluble MUC16 was a potent inhibitor of NK cell function. A manuscript on this topic is now published in the journal Gynecologic Oncology (appendix 1). The funding from the DOD has been gratefully acknowledged in this manuscript.

We have now obtained extensive data that MUC16 coats the immune cells isolated from patients with ovarian cancer (Fig. 2). A majority of the NK cells from the peripheral blood and the peritoneal fluid carried MUC16 on their surface (data not shown). The immune cells from the patients did not express endogenous MUC16 as per our RT-PCR analysis (Fig. 3). This observation has been made with immune cells from the peripheral blood and the peritoneal fluid of over 15 patients. Binding of MUC16 to the NK cell surface is associated with a decrease in the cell surface expression of CD16 and NKp46 on these cells (data not shown). Exactly how MUC16 can affect the expression of these markers on the NK cells is under extensive investigation in the PI's laboratory. A manuscript on this topic is under construction. The funding from the DOD will be acknowledged in this manuscript. This data on the NK cells has been presented at several international level scientific conferences. The abstracts for these meetings are included in the appendix. Funds from the DOD were acknowledged in these presentations. One of the abstracts was selected for an oral plenary presentation at the Society for Gynecologic Oncologists annual meeting in 2005. A second abstract was selected for a poster discussion session at the International Society for Biological Therapy meeting in 2005.

In the second specific aim of the grant we proposed to study the effect of cell surface associated MUC16 on the proper functioning of the NK cells. We have now developed an excellent in vitro model that will help us immensely in achieving the goals of this specific aim. We have now established that a NK cell leukemia cell line, NKL, can selectively lyse a higher proportion of the ovarian tumor cells that have been specially engineered to not express MUC16. Matching cell lines that continue to express MUC16 are not lysed to the same extent. Although in our initial experiments we utilized only microscopic techniques to quantify the killing of the ovarian tumor cells by the NKL cells (Fig. 4), we have now developed a flow cytometry based quantitative assay that perfectly corroborates the results we obtained in our initial studies (data not shown). We will now extensively utilize this assay to achieve the goals of specific aim 2.

Role of MUC16 on T cells. We have not directly tested the effect of MUC16 on human T cells. However we have now obtained data demonstrating that MUC16 can bind to CD209, a receptor on the dendritic cells (data not shown). The dendritic cells are very effective in presenting antigens to the T cells. It remains to be seen if the binding of MUC16 to the dendritic cells has any effect on antigen presentation. The studies proposed for specific aim 3 of the grant will take into account the data on the dendritic cells.

Role of MUC16 in metastasis of ovarian tumors. We are in a very exciting collaboration with Dr. Ira Pastan at the National Cancer Institute. Our data suggests that MUC16 plays a major role in facilitating the peritoneal metastasis of the ovarian tumors (data not shown). A manuscript on this topic has been submitted to the journal Molecular Cancer and the funding from the DOD has been acknowledged.

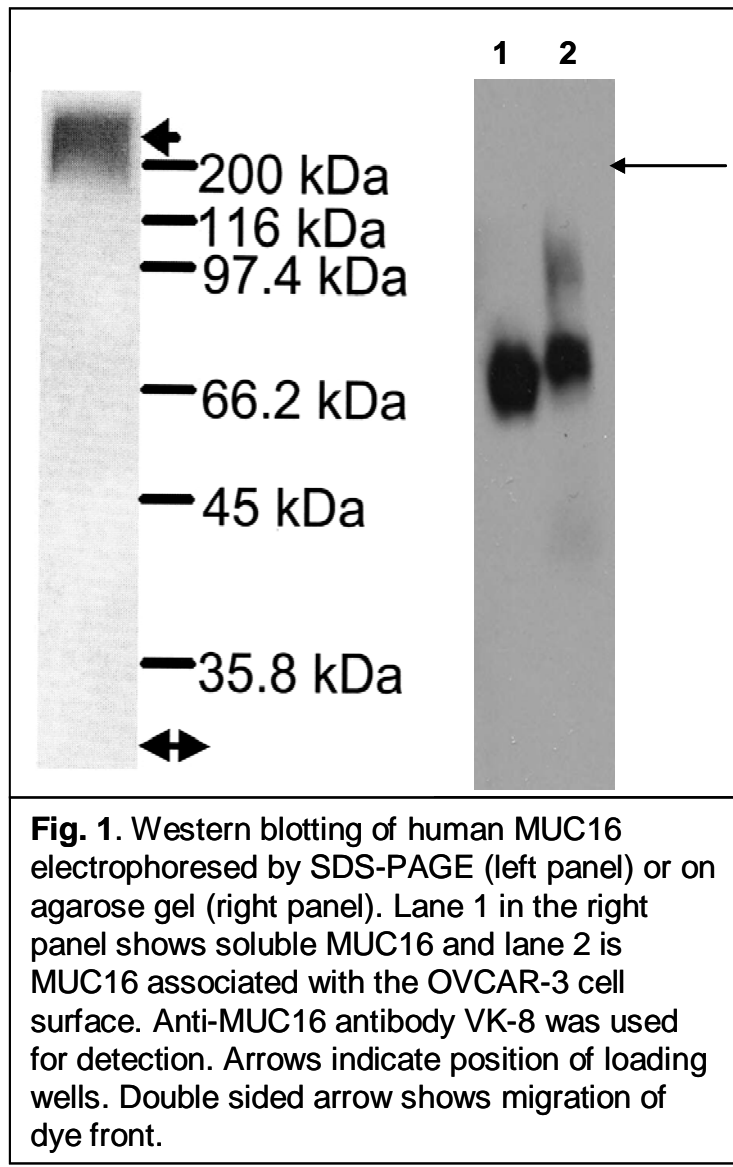
RESEARCH ACCOMPLISHMENTS:

Due to the delay in the initiation of this grant we do not have any major research accomplishments to report at this time. The advances we have made in understanding the structure and biology of MUC16, which have a significant impact on the proposed studies, are reported above.

REPORTABLE OUTCOMES: One paper and several abstracts on the studies briefly summarized above have been generated. Copies of these materials are provided in the appendix.

CONCLUSION: Overall we believe that MUC16 is an extremely important molecule that facilitates the pathogenesis of ovarian tumors. Exhaustive studies on the structure and biology of this molecule may lead to the development of novel treatment strategies for ovarian cancer.

SUPPORTING DATA



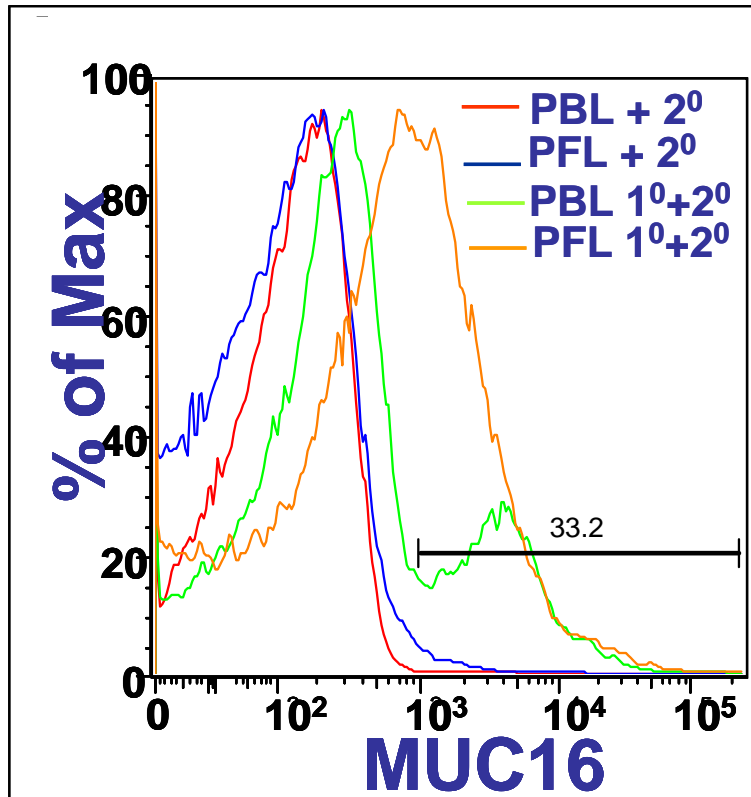
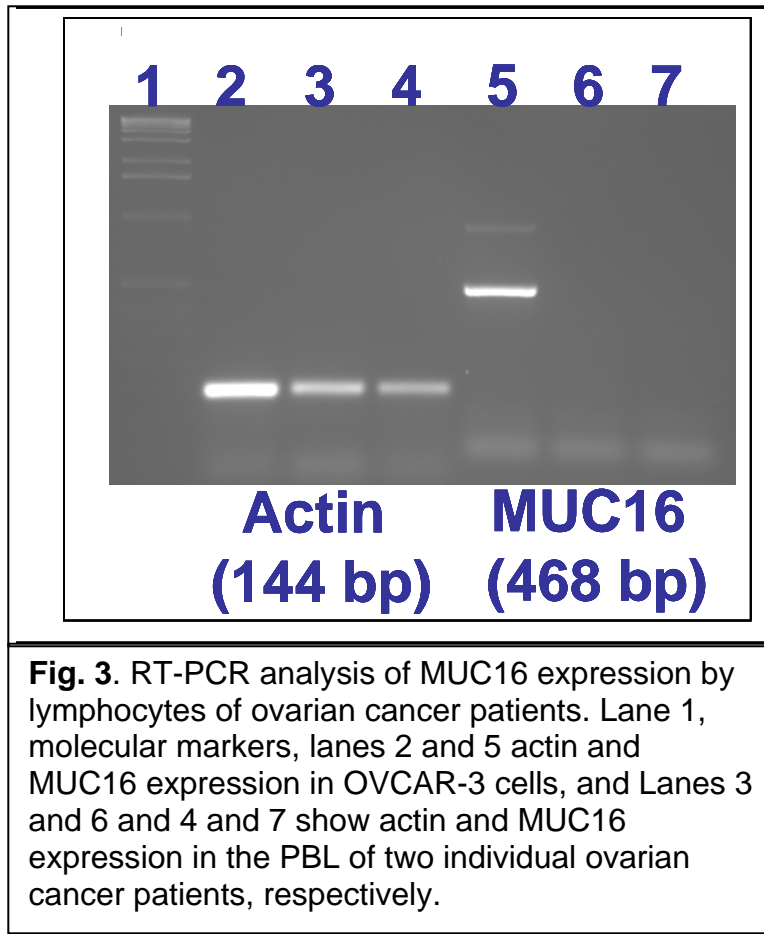


Fig.2. MUC16 is present on the surface of lymphocytes of ovarian cancer patients. VK-8 (1⁰) was used to detect MUC16 on the peripheral blood (PBL) and peritoneal fluid (PFL) derived lymphocytes by flow cytometry. A FITC labeled goat anti-mouse IgG was used as the secondary antibody (2⁰).



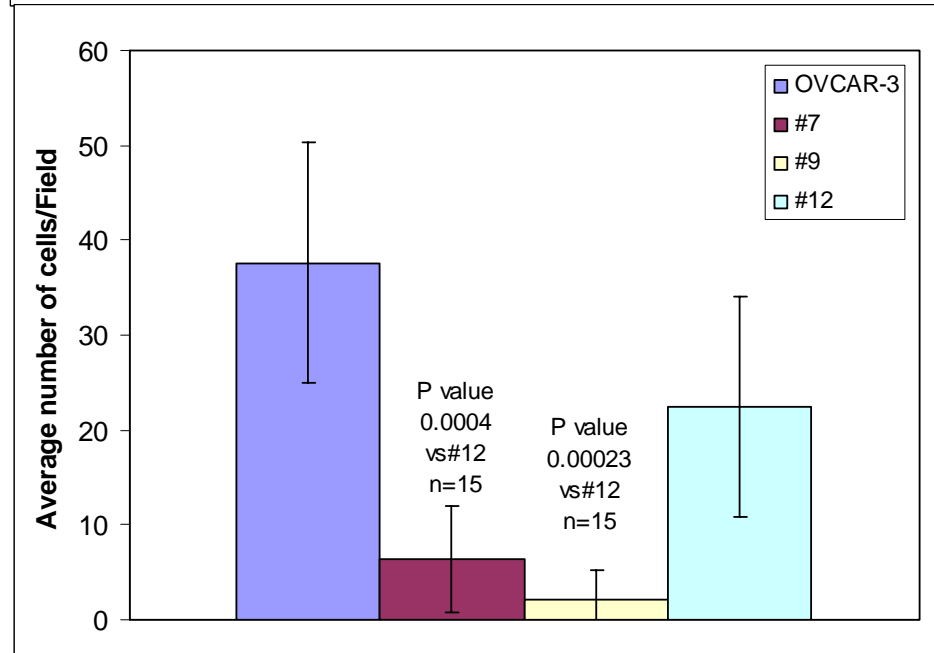
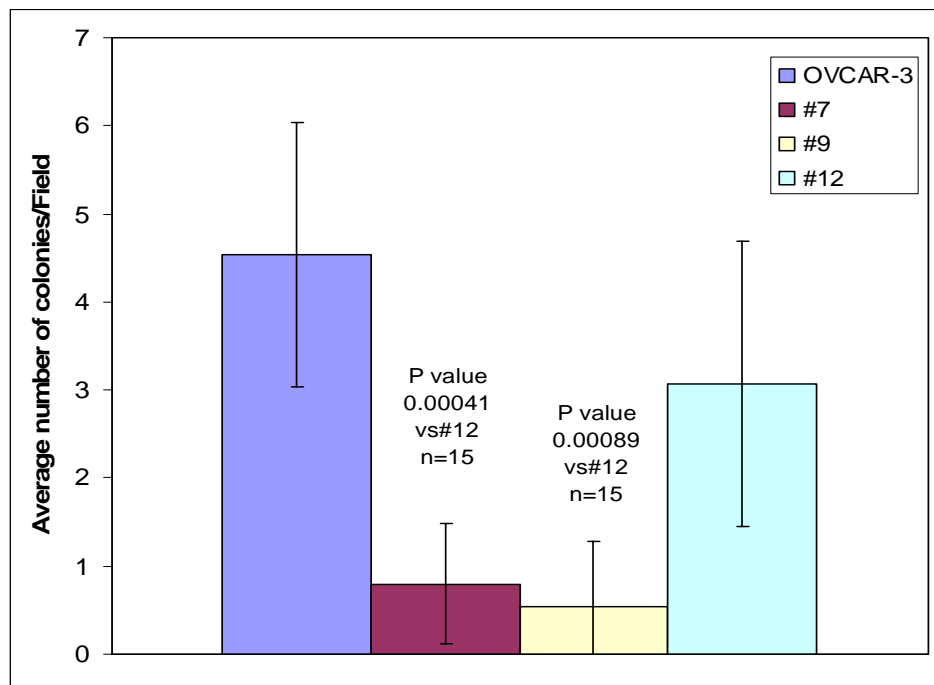


Fig. 4. Increased lysis of MUC16 negative ovarian tumor cells by NKL. NKL cells were mixed with adherent OVCAR-3 and OVCAR-3 derived sublines #7, #9, and #12 at 122:1 effector:target ratio. The #7 and #9 express no MUC16 on their surface whereas #12 express normal levels of MUC16 similar to OVCAR-3. Upper panel shows the number of surviving colonies and lower panel shows the total number of live cells after a 24 h co-incubation.

Appendix

Selected for Oral Presentation

Potent Suppression of Natural Killer Cell Response Mediated by the
Ovarian Tumour Marker CA125

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Objectives: The identification of the CA125 gene has provided great impetus for functional and biochemical characterization of this molecule. We have recently characterized the glycans that are expressed on CA125 using ultra-sensitive mass spectrometric analysis. This analysis indicates the presence of specific oligosaccharide sequences on CA125 that have previously been associated with altering the function of human natural killer (NK) cells. The current study was undertaken to determine the ability of CA125 to modulate NK cell mediated cytotoxicity.

Methods: CA125 was isolated from OVCAR-3 cells. The purity of the CA125 preparation was determined by ELISA and by performing mass spectrometric analysis. NK cells were isolated from peripheral blood of healthy donors. The NK cells were treated with CA125 and standard cytotoxicity assays were performed using ⁵¹Cr labeled K562 cells as targets. The expression of cell surface and intracellular markers on NK cells was determined by either flow cytometry or western blot analysis.

Results: CA125 was found to be a potent inhibitor of NK cell mediated cytotoxicity. This suppressive effect was observed at CA125 concentrations between 10,000-100,000 U/ml. NK cells derived from 15 healthy donors when treated with 50,000 U/ml of CA125 were routinely found to exhibit 60-70% inhibition of K562 cell lysis. This suppression was found to be independent of the sex or the age of the blood donors. CA125 was also a potent inhibitor of IL-2 stimulated NK cells. This inhibition of the NK cell function by CA125 was not due to reduced proliferation or apoptosis. Western blot and flow cytometry analysis indicated that CA125 did not induce any major changes in the expression of the cell signaling molecules p56lck, phospholipase C γ 1, ZAP70 or CD3 ζ . CA125 did however induce major downregulation of CD16 although no alterations in the expression levels of CD56 and CD94/NKG2A were observed.

Conclusions: To date CA125 has been extensively studied as a marker for epithelial ovarian cancer. Our ongoing research and recent work performed by other laboratories highlights the potential physiologic role of this mucin. Based on the data presented here, it is likely that the tumor derived CA125 acts as a suppressor of the immune response that is directed against the cancer. (Supported by grants to M.S.P. from the Jeffress Trust, Elsa U. Pardee Foundation and the Department of Defense).

Selected for Poster Discussion Session

The role of the ovarian tumor marker CA125 (MUC16) in the suppression of human immune responses

Jennifer Belisle¹, Jennifer Arens¹, Martine Migneault², Claudine Rancourt², Mildred Felder¹, Joseph Connor¹, and Manish S. Patankar¹

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The epithelial ovarian cancer (EOC) marker CA125 is a peptide epitope that is expressed on the mucin MUC16. Clinical trials are currently underway to determine the efficacy of anti-CA125 immunotherapy for the treatment of EOC. Recent identification of the MUC16 gene and our analysis of the oligosaccharides expressed on this mucin have provided renewed impetus to investigate its biological role in ovarian tumor progression. While our current data suggests that MUC16 may facilitate tumor metastasis, we have also made the observation that MUC16-treated peripheral blood NK cells derived from healthy donors exhibit a severe loss of cytotoxic function. A similar loss of cytotoxicity versus the erythroleukemia target cell line K562 is seen when IL-2 stimulated LAK cells and the highly cytotoxic NK cell leukemia cell line NK-92 are incubated with MUC16. Loss of cytotoxic function is associated with a significant downregulation of CD16, an activating receptor present on NK and LAK cells that binds to the Fc portion of antibodies and mediates antibody dependent cell mediated cytotoxicity. MUC16 mediated decrease in CD16 correlates with the significant downregulation of this receptor in the NK cells derived from patients with EOC [Lai et al, (1996) *Clin. Cancer Res.* **2**, 161-173]. We have confirmed this observation and have further shown that NK cells from patients with EOC also show a similar decrease in the expression of NKP46, an activating natural cytotoxicity receptor expressed primarily on human NK cells. In this context it is important to note that lymphocytes from EOC patients carry MUC16 on their surface. In our on-going studies we are trying to determine if the MUC16 present on the lymphocyte cell surface is the result of endogenous production of the mucin by these cells or if MUC16 expressed by the ovarian tumor cells specifically binds to the immune cells. Experiments studying the kinetics of MUC16 mediated inhibition of NK cytotoxicity and CD16 downregulation suggest that these effects are realized only after a 72 h incubation of the cells with the mucin. These data are consistent with our recent observation that MUC16⁻ clones of the ovarian tumor cell line OVCAR-3 do not exhibit higher susceptibility to NK cell mediated lysis in a standard 4h chromium release assay. Overall these observations suggest an important role for MUC16 in modulating anti-tumor immune responses and are likely to have considerable impact on our understanding of the biology of EOC and the development of immunotherapeutic approaches for the treatment of this disease.

Both abstract selected for poster presentation

Cell Surface Expression of MUC16 Prevents Effective Lysis of OVCAR-3 Cells by the Natural Killer (NK) Cell Leukemia Cell Line, NKL- A Model to Study NK cell Dysfunction in Ovarian Cancer Patients

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Objectives: MUC16 inhibits the cytolytic function of human NK cells. The NK cells represent only 5-10% of the total human lymphocyte population, hence limiting our ability to extensively study the suppressive mechanisms evoked by MUC16 in this cell type. Therefore our goal was to utilize the NK leukemia cell line NKL to establish a more convenient model to study the effect of MUC16 on NK cells.

Methods: OVCAR-3 cells that expressed an intracellular single chain fragment of the anti-CA125 antibody VK-8 were produced. Spent media from these cells was tested for the expression of secreted MUC16. Lack of cell surface MUC16 in these cells was confirmed by flow cytometry. These MUC16^{neg} and the parental OVCAR-3 cells were plated in 24 well plates. To confluent cultures were added the NKL cells. Surviving tumor cells and colonies were counted at various time points. The MUC16^{pos} cells that survived this treatment were cultured and treated again with the NKL cells. MUC16 expression on the NKL resistant cells was determined by flow cytometry.

Results: The NKL cells are highly cytolytic and were therefore able to kill approximately 70% of the MUC16^{pos} OVCAR-3 cells. The surviving 30% cells when washed to remove the NKL cells and cultured further in fresh media were not susceptible to lysis by the NKL cells. These cells when analyzed by flow cytometry exhibited approximately 20-30% more MUC16 on their cell surface than the NKL-untreated OVCAR-3 cells. The NKL cells almost completely lysed the MUC16^{neg} OVCAR-3 cells. The miniscule number of MUC16^{neg} cells that did survive this treatment were unable to proliferate upon further culture.

Conclusions: The results clearly indicate that high surface expression of MUC16 renders the ovarian tumor cells resistant to killing by the NKL cells. This study concurs with our original observation on the suppressive effect of MUC16 on human NK cells. The NKL cells can therefore be used as an effective model to study MUC16 induced NK cell dysfunction.

Phenotypic and Functional Analysis of Natural Killer Cells in Patients with Epithelial Ovarian Cancer

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Objectives: The ovarian cancer marker MUC16 (CA125) inhibits human natural killer (NK) cell function and alters the expression of activating receptors on this cell type. The purpose of this study is to determine if similar phenotypic differences are also observed in NK cells derived from the peripheral blood (PB) and peritoneal fluid (PF) of patients with epithelial ovarian cancer (EOC).

Methods: Lymphocytes from the PB (PBL) and PF (PFL) were isolated from patients with advanced stage EOC. The cells were labeled with a panel of antibodies against human CD3, CD4, CD8, CD16, CD19, CD45, CD56, CD94/NKG2A, and NKp46. The labeled cells were analyzed by multi-color flow cytometry. The expression of the cell surface markers was analyzed using FlowJo (Treestar) software. The MUC16 levels in the PB and PF were determined by the clinical assay for CA125. PBL from healthy donors were incubated with PF from EOC patients for 72 h, and the phenotypic changes in the NK cells were also monitored by flow cytometry.

Results: We report a reduced expression of CD16 on the PFL of patients with EOC as compared to PBL of the same patients. Downregulation also occurs with NKp46, which is an activating receptor found on NK cells. Furthermore, analysis of healthy donor PBL incubated with PF shows a shift toward the CD16^{dim}/CD56⁺ phenotype, which differs from the CD16⁺/CD56⁺ phenotype of NK cells in the peripheral blood of normal individuals.

Conclusions: Increased downregulation of CD16 and NKp46 in the PFL compared to the PBL of EOC patients was observed. PBL from healthy donors when cultured in PF also exhibited a similar decline in the expression of these NK cell activation receptors. In *in vitro* experiments we have shown that MUC16 causes downregulation of CD16 on healthy donor derived PBL. The increased downregulation of the NK cell activation receptors on the PFL compared to the PBL is congruent with the 10-20-fold higher concentrations of MUC16 in the PF as compared to the serum of EOC patients. This observation suggests a direct role for MUC16 in modulating NK cell responses.

Paper published in the journal Gynecologic Oncology

Potent suppression of natural killer cell response mediated by the ovarian tumor marker CA125

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Abstract

Objectives. CA125 expresses specific oligosaccharides that can inhibit the cytotoxicity of human natural killer (NK) cells. The current study was undertaken to determine the ability of CA125 to modulate NK cell-mediated cytotoxicity.

Methods. CA125 was isolated from OVCAR-3 cells and its purity was determined by ELISA and ultra-sensitive mass spectrometric analysis. Peripheral blood-derived NK were treated with CA125 and standard cytotoxicity assays were performed using ⁵¹Cr-labeled K562 cells as targets. The expression of cell surface and intracellular markers on NK cells was determined by either flow cytometry or Western blot analysis.

Results. NK cells incubated with CA125 for 72 h exhibited a 50–70% decrease in the lysis of K562 targets. Incubation with CA125 for 4 h and 24 h had no effect on NK-mediated cytotoxicity. Inhibition of NK function was observed at CA125 concentrations (10,000–100,000 U/ml) that are expected to be significantly lower than those observed in the tumor microenvironment. Co-stimulation with IL-2 did not abrogate the NK inhibitory response of CA125. CA125 did not reduce proliferation or induce apoptosis of NK cells and alter the expression of p56lck, phospholipase Cγ1, ZAP70, or CD3ζ. CA125 did, however, induce major downregulation of CD16 and minor decrease in expression of CD94/NKG2A.

Conclusions. Our ongoing research and recent work performed by other laboratories highlights the potential physiologic role of this mucin. Based on the data presented here, it is likely that the tumor-derived CA125 acts as a suppressor of the immune response that is directed against the ovarian tumors.

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Keywords: CA125; Ovarian cancer; Natural killer cell; Mucin; CD16; Immune-suppression

Introduction

CA125, also designated MUC16, is a mucinous glycoprotein first detected by Bast and colleagues using the

mouse monoclonal antibody OC125 [1,2]. CA125 is produced in many human cancers, but is best known for its utility in monitoring the progression of EOC⁵, especially after therapeutic intervention [3]. Serum levels of CA125 greater than 35 U/ml are diagnostic for the recurrence of EOC under specific clinical conditions [4]. However, levels as high as 41,678 and 744,770 U/ml have been reported in the ovarian ascites and cyst fluids, respectively, in patients with serous type ovarian carcinomas [5].

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Since CA125 is expressed by epithelial ovarian tumors, ongoing clinical trials are investigating the use of this molecule as a target for immunotherapy in patients with EOC. Administration of the immunoscintigraphic monoclonal antibody B43.13 increases the survival time of EOC patients [6,7]. Induction of humoral response to CA125 increases the mean survival time in EOC patients from 5.3 ± 4.3 to 19.9 ± 13.1 months [8].

While the potential of CA125 as a diagnostic marker and an antigen for anti-tumor therapy for EOC has been investigated, a clear understanding of the physiological role of this mucin is not known. Recent advances in characterizing the structure of CA125 have provided fresh impetus to study the role of this mucin during ovarian cancer progression.

CA125 is an extremely complex molecule [9] composed of an N-terminal domain, a tandem repeat region, and a short cytoplasmic tail [10,11]. The core protein backbone of CA125 has an average mass of 2.5 million Da. The carbohydrate content of CA125 is estimated to be between 24 and 28% [9,12], suggesting that the average molecular weight of the glycosylated mucin exceeds 3.5 million Da. Both N- and O-linked oligosaccharides are expressed on CA125 [13].

The bisecting type biantennary oligosaccharide, a major N-linked glycan expressed on CA125 can inhibit the cytotoxic responses of human natural killer (NK) cells [14,15]. The NK cells are an important arm of the innate immune response and can react against virally infected cells and tumors. A majority of the NK cells in human peripheral blood express the Fc receptor CD16 and the cell adhesion molecule CD56 [16]. In addition, the human NK cells also express activating and inhibitory receptors [17]. A delicate interplay between these activating and inhibitory receptors and the ligands they bind determines the cytolytic response of the NK cells [18].

Since the NK-suppressive bisecting-type N-linked glycans are highly expressed on CA125, we hypothesized that this EOC marker may also act as a modulator of NK cell cytotoxicity. The data reported here clearly indicate that CA125 is a potent inhibitor of NK cell-mediated lysis of the K562 cells, an erythroleukemia cell line. Using flow cytometry, we further demonstrate that this inhibition in NK cell function is not due to necrosis or apoptosis of these effector cells but instead is correlated to a severe reduction in CD16 expression on the cell surface. The biological and clinical significance of this observation is discussed below.

Materials and methods

The epithelial ovarian tumor cell line, OVCAR-3, and the NK tumor cell line, NK-92MI, were purchased from ATCC. Fetal calf serum (FCS) and RPMI-1640 were from Atlanta Biologicals and Cellgro, respectively. Reagents for

gel electrophoresis and Western blot analysis were purchased from BioRad. Immunotech was the supplier of the Z199 (anti-CD94/NKG2A), anti-CD56, anti-CD16, and anti-CD3 ζ antibodies and anti-p56lck was obtained from Upstate USA. Anti-ZAP70 and anti-phospholipase-C γ 1 (PLC- γ 1) antibodies were from BD Biosciences. Horseradish peroxidase (HRP) and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG secondary antibodies were from Amersham and Jackson Immunoresearch Laboratories, respectively. A Becton Dickinson FACSCalibur[®] instrument was used to perform the flow cytometric analysis. Statistical analysis was performed using paired two-tailed Student's *t* test unless noted otherwise.

Isolation and characterization of CA125 from OVCAR-3 cells

CA125 was isolated from OVCAR-3 cells as previously described [13,19]. All preparations were subjected to proteomic analysis to determine their purity using criteria established in our prior study [13]. All of the major tryptic peptides were sequenced and found to be associated with CA125 and were consistent with the established protein sequence of this mucinous glycoprotein [11,13]. These results indicate that the preparations employed in this study were very highly enriched in CA125.

In addition, a commercially available ELISA (Glycotect) was employed to determine the specific activity of the isolated CA125 samples. Only those samples that had a specific activity of 2 million units of CA125 per mg of total protein were analyzed for their effect on human NK cell function.

NK, LAK, and NK-92MI cell cytotoxicity assays

NK cells were isolated from peripheral blood of healthy donors (designated as D1, D2, D5, D6, D7, D8, and D11) either by the classical negative selection methodology [20] or by using the RosetteSep isolation kit (Stem Cell Technologies). Purity of the isolated NK cells was determined by flow cytometry. On average, between 70 and 90% of the isolated cells were CD3⁺/CD16⁺/CD56⁺, the typical phenotype of human NK cells. NK cells were stimulated with interleukin-2 (IL-2; 1000 U/ml) to generate the LAK cells. The RPMI-1640 media containing 10% FCS was used to culture the NK and LAK cells and the NK-92MI cells were maintained in MyeloCult H5100 (StemCell Technologies) media.

The effector cells (2.5×10^6 cells/ml) were incubated in the presence or absence of CA125 for the designated time intervals prior to conducting the lysis assays. The NK, LAK, or NK-92MI cells were co-incubated for 4 h with 5×10^3 [⁵¹Cr]-labeled K562 cells at appropriate effector:target (E:T) ratios. Assays were performed in a total volume of 200 μ l, as described previously [21].

Following incubation, a 100- μ l aliquot was withdrawn from the wells and the amount of radioactivity was determined using a gamma counter. Higher lysis of the K562 cells results in increased ^{51}Cr counts in the supernatant media in this cytotoxicity assay. Proliferation of LAK and NK-92MI cells was determined by performing the CyQuant[®] (Molecular Probes) assays using the protocol suggested by the manufacturer.

Measurement of apoptosis by flow cytometry

Annexin V assays were performed to determine if CA125 induced apoptosis in the NK cells. Briefly, the NK cells were incubated with 0, 10, and 50 kU/ml CA125 for the appropriate time intervals. Corresponding control and test NK cells were stained with FITC-labeled annexin V (Oncogene) as per the manufacturer's protocol. Immediately prior to analysis, propidium iodide (PI) was added to all of the samples and the percentage of cells undergoing cell death by apoptosis (those binding to annexin V) and necrosis (cells positive for PI) were determined by flow cytometry. By this protocol, cells that are in advanced stages of apoptosis are also detected as annexin V positive cells that are also stained with PI.

Effect of CA125 on the expression of cell surface receptors and intracellular signaling molecules in NK cells

To determine the effect of CA125 on the expression of CD16, CD56, CD94/NKG2A, NK cells were treated in the presence or absence of CA125 and washed twice with PBS-BSA. The cells were labeled with the corresponding fluorophore conjugated primary antibodies and analyzed by flow cytometry. The mean fluorescence intensity (MFI) was determined and is presented as the geometric mean of CD16 or CD94/NKG2A positive cells.

To determine the changes in the expression of CD3 ζ , NK cells (1×10^6 cells/150 μ l) were washed with PBS-BSA and then permeabilized with 1% digitonin in PBS-BSA for 1–2 min. The phycoerythrin-labeled anti-CD3 ζ antibody was added and the cells were incubated for 20 min on ice. Cells were washed three times with PBS-BSA and analyzed by flow cytometry.

To monitor for changes in expression of ZAP-70 and PLC- γ 1, NK cells (1×10^6 /150 μ l) were fixed in phosphate buffered saline containing 1% paraformaldehyde for 10 min at room temperature. The cells were centrifuged to remove the buffer and permeabilized for 10 min at room temperature with 200 μ l of PERM2 (BD Biosciences). After removing the permeabilization buffer and washing twice with PBS-BSA, the cells were incubated with anti-ZAP-70 (1:500 dilution) and anti-PLC- γ 1 (1:100 dilution) antibodies for 20 min on ice. Excess primary antibodies were removed by washing with PBS-BSA and the cells were labeled for 20 min on ice with FITC conjugated secondary antibody and analyzed by flow cytometry.

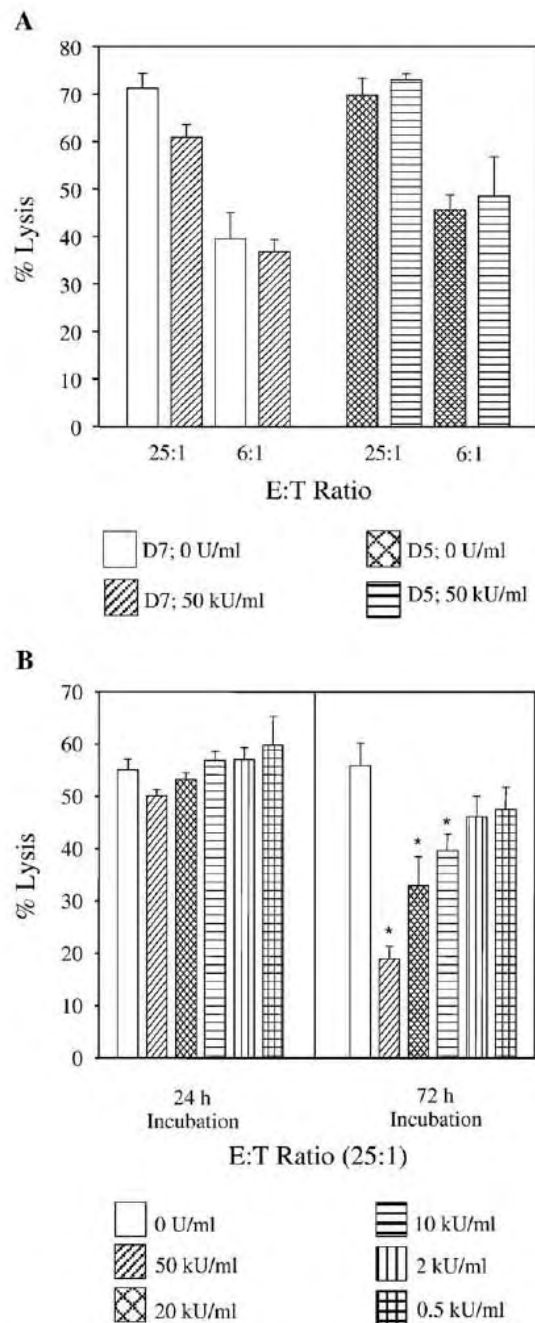


Fig. 1. Effect of CA125 on NK cell-mediated cytotoxicity. NK cells were isolated from the peripheral blood of healthy male and female donors [20]. Standard cytotoxicity assays were performed in triplicate using ^{51}Cr -labeled K562 cell targets (5×10^3) as described previously [21]. The K562 cells were mixed with NK cells derived from donors D7 and D5 (A) immediately after the addition of CA125 (50 kU/ml). Corresponding control incubations were performed in the absence of CA125 (0 kU/ml). The lysis assays were performed at 25:1 and 6:1 effector:target (E:T) ratios. To determine if prolonged incubation with CA125 had any effect on K562 cell lysis, NK cells (5×10^5 cells/200 μ l) derived from donor D8 were preincubated with the indicated concentrations of CA125 for 24 h or 72 h (B). The NK cells were then mixed with K562 target cells at 25:1 E:T ratio for the standard lysis assay. The data shown here are representative of identical experiments performed using NK cells derived from at least five different donors. **P* values are <0.01; *P* values for all other measurements in this figure are >0.05.

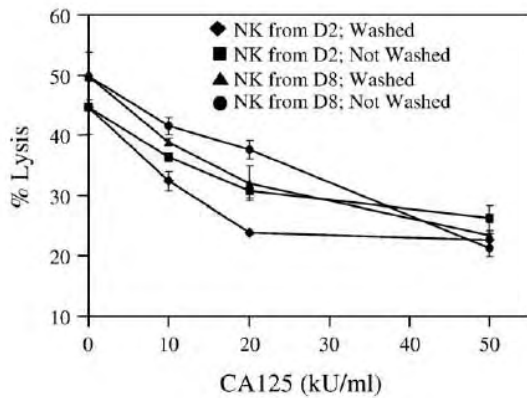


Fig. 2. Inhibition of NK cell-mediated cytotoxicity even after removal of CA125. NK cells (5×10^5 cells/200 μ l) from donors D2 and D8 were incubated with 0, 10, 20, and 50 kU/ml CA125. After a 72-h incubation, one half of the cells were washed 3 times and cultured for an additional 24 h in media lacking CA125 to generate washed cells. The remaining NK cells were incubated in the presence of CA125 for an additional 24 h without washing (not washed). The NK cells were then mixed with [51 Cr]-labeled K562 cells to perform the standard lysis assay at 25:1 E:T ratio. This experiment was performed on NK cells derived from at least three donors. Representative results are presented. *P* values for all points >0.05 .

Western blot analysis of p56lck

NK and LAK cell lysates were prepared as described in a previous study [22]. The cell lysates (10 μ g/lane) were separated under denaturing conditions (SDS-PAGE) on 7.5% separating and 3% stacking gels [22]. The proteins were transferred to nylon membranes using BioRad minigel apparatus. The nylon membranes were probed with anti-p56lck. The HRP-labeled secondary antibody was employed for the detection of the p56lck bands using chemiluminescence reagents (Amersham).

Results

Effect of CA125 on NK cell cytotoxicity

Standard cytolytic assays were performed to determine the effect of CA125 on the ability of human NK cells to lyse the chromium-labeled erythroleukemia target cell line K562. Incubation of NK cells with CA125 during the standard 4-h lytic assay did not affect their cytotoxic activity (Fig. 1A). We considered the possibility that tolerance could be induced after prolonged exposure of the NK cells to CA125. Therefore, NK cells were incubated in the presence of CA125 for 24 h and 72 h (Fig. 1B) prior to performing the cytotoxicity assays. No effect was observed after 24 h, but there was significant concentration-dependent inhibition of NK cell-mediated killing of K562 cells after a 72-h preincubation. This result indicates that chronic exposure of NK cells to CA125 induces tolerance to the K562 targets. This

inhibitory effect of CA125 was observed with NK cells regardless of the sex or the age of the donors (data not shown).

Inhibition of NK cell cytotoxicity is observed even after removal of CA125 from the culture media

The functional capacity of lymphocytes derived from the tumor microenvironment is compromised [23]. We therefore decided to test if CA125 could induce prolonged suppression of NK cell-mediated cytotoxicity even after removal of the mucin from the culture media. Test NK cells were exposed to different concentrations of CA125 for 72 h and then washed and incubated in media lacking CA125 for an additional 24 h. Control NK cells were incubated for 96 h in CA125 without washing. Com-

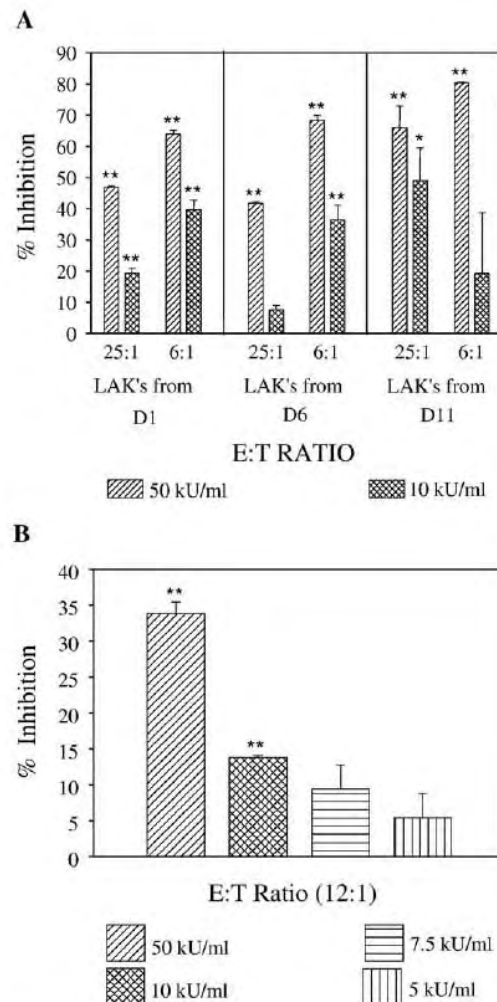


Fig. 3. CA125 inhibits the lytic activity of LAK and NK-92MI cells. LAK cells (A) from donors D1, D6, and D11 and NK-92MI (B) cells were incubated in the presence or absence of CA125 at the indicated concentrations. Lytic assays were performed in triplicate after 72-h incubation using the E:T ratio indicated. *Mean of two readings; ***P* values for corresponding differences in the percent lysis <0.05 .

parable inhibition of the cytolytic responses was observed with the test and control NK cells (Fig. 2) indicating that the suppressive effect of CA125 on NK cell function is maintained for at least 24 h after removal of this mucin from the culture media.

Effect of CA125 on LAK and NK92-MI cell activity

Incubation of NK cells with the cytokine IL-2 transforms them into LAK cells that exhibit increased ability to lyse tumor cell targets [24]. We therefore determined if CA125 could also inhibit LAK cells. NK cells from donors D1, D6, and D11 were simultaneously treated with IL-2 and 0, 10, and 50 kU/ml CA125 for 72 h prior to performing standard cytotoxicity assays. Efficient lysis of the K562 targets (60–80% at 25:1 effector:target ratio; 30–40% at 6:1 effector:target ratio) is observed with LAK cells that were not treated with CA125. However, as shown in Fig. 3A, CA125 is a potent inhibitor of LAK cell cytotoxicity at the same concentrations required for the suppression of NK cell-mediated response. This suppression of cytotoxicity was not

due to inhibition of proliferation of the LAK cells (data not shown).

NK-92MI cells are NK tumor cells that constitutively express IL-2 [25] and exhibit significantly higher cytotoxicity against various tumor targets as compared to LAK cells. On average, 70–80% lysis of K562 targets was observed with NK-92MI cells that were not treated with CA125. A 33% inhibition of K562 cytotoxicity was observed with NK-92MI cells that were incubated with 50 kU/ml CA125 (Fig. 3B). The proliferation of the NK-92 cells was not adversely affected (data not shown).

CA125 does not induce apoptosis in NK cells

Trypan blue dye exclusion assays conducted on NK cells exposed to CA125 for 72 h did not indicate any effect on cell viability (data not shown). Since prolonged exposure of NK cells to CA125 is required to induce tolerance, we also determined if these lymphocytes were undergoing apoptosis. NK cells from donor D5 were treated with 0, 10, and 50 kU/ml CA125 for 72 h and

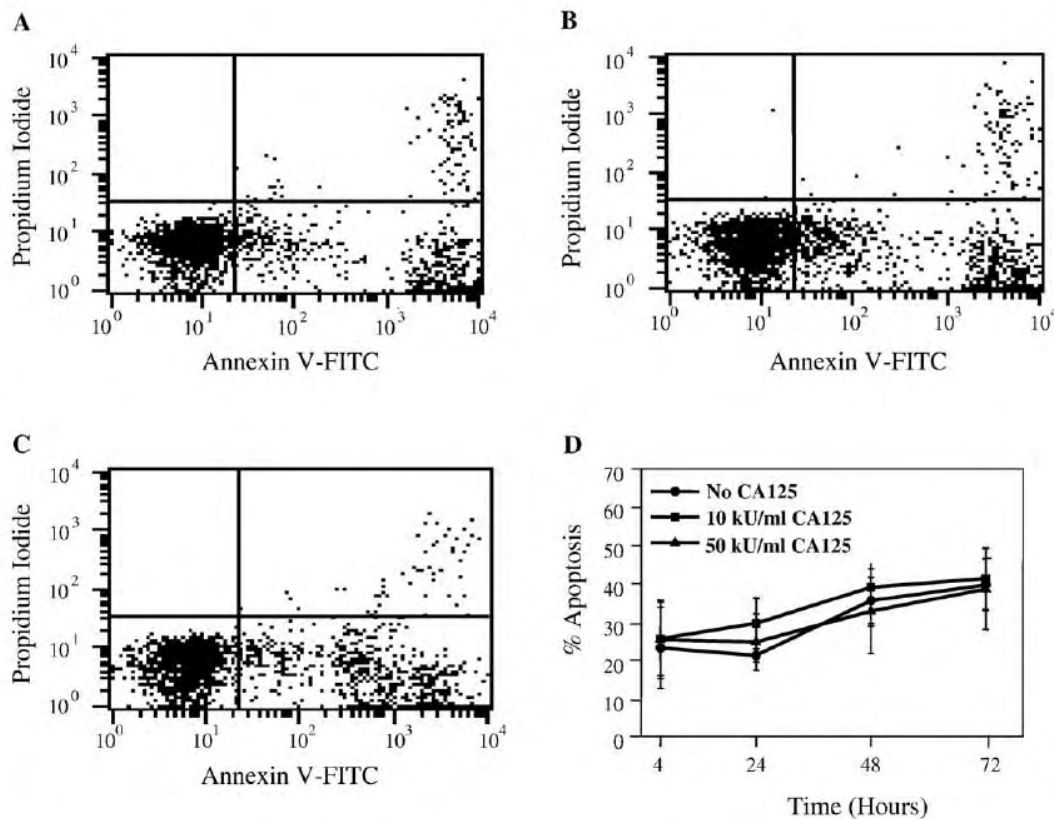


Fig. 4. CA125 does not induce necrosis or apoptosis of NK cells. NK cells derived from donor D5 were incubated with 0, 10, and 50 kU/ml CA125 for 72 h. The percentage of cells undergoing apoptosis or necrosis was determined by flow cytometry analysis using a standard annexin V assay [48]. Dot plots for these experiments are shown (A–C). Under these conditions, cells undergoing necrosis and apoptosis appear in the upper and lower right quadrants, respectively. For this set of experiments, the percentage of NK cells undergoing apoptosis after exposure to 0 (A), 10 (B), and 50 (C) kU/ml of CA125 for 72 h was 26.6, 26.7, and 23.3%, respectively. Composite data for time-course studies with NK cells from three different donors (D1, D5, and D8) that were treated with 0, 10, and 50 kU/ml CA125 are shown in panel D. NK cells from the three donors were treated separately with the designated concentrations of CA125 for either 4, 24, 48, and 72 h. Each point in the plot represents an average of the percent apoptotic cells that was obtained for each individual donor.

monitored for apoptosis by flow cytometry using standard annexin V binding assays (Figs. 4A–C). This analysis did not reveal any major effect of CA125 on the apoptosis of the peripheral blood-derived NK cells. A composite analysis of the effect of 0, 10, and 50 kU/ml of CA125 on the apoptosis of NK cells derived from three different donors (D1, D5, and D8) following preincubation for 4, 24, 48, and 72 h further confirmed this initial observation (Fig. 4D).

Effect of CA125 on p56lck, CD3 ζ , ZAP70, and PLC- γ 1 expression

Tumor-infiltrating NK cells isolated from patients with EOC also exhibit a severe downregulation of proliferative responses [22]. This decrease in functionality is also associated with reduced expression of the key signaling molecules p56lck, CD3 ζ , and ZAP70 [22]. CA125 did not significantly downregulate p56lck in either NK or LAK cells (Fig. 5A). However, we observed a marginal decrease in the intensity of anti-CD3 ζ expression,

although the frequency of CD3 ζ ⁺ cells increased slightly (Fig. 5B). We also did not observe any significant change in the expression of ZAP70 (Fig. 5C) and PLC- γ 1 (Fig. 5D), crucial enzymes involved in lymphocyte activation [26], in the NK cells that were exposed to CA125 for 72 h.

Effect of CA125 on CD16 expression

Another phenotypic alteration associated with NK cells isolated from ovarian tumor microenvironment is an 86% decrease in Fc receptor expression detected by anti-CD16 monoclonal antibodies [22]. In the current study, incubation of CA125 with NK cells for 72 h resulted in a 40–70% decrease in the expression of CD16 based on the difference in MFI of anti-CD16 monoclonal antibody binding (Figs. 6A–C). Exposure to CA125 also decreased the expression of CD16 on LAK cells in an equivalent fashion (data not shown). Progressive loss of CD16 is observed as exposure of the NK cells to CA125 is increased from 24 to 72 h (Figs. 6D and E).

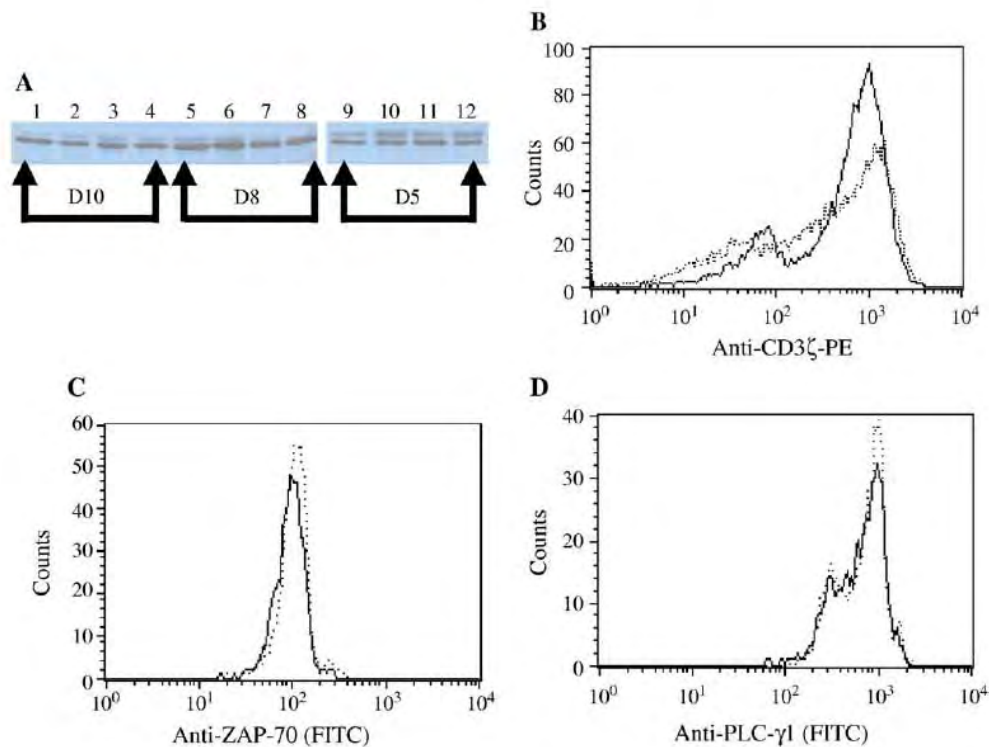


Fig. 5. Incubation with CA125 marginally affects the expression of CD3 ζ but not p56lck, ZAP70, and PLC- γ . NK cells (lanes 1, 3, 5, 7, 9, and 11) and LAK cells (lanes 2, 4, 6, 8, 10, and 12) isolated from donors D5, D8, and D10 (indicated at the bottom of panel A) were treated in the absence (lanes 1, 2, 5, 6, 9, and 10) or presence (lanes 3, 4, 7, 8, 11, and 12) of 50 kU/ml of CA125. Cell lysates obtained after treatment were analyzed for p56lck expression by Western blotting [22]. Lysate from Jurkat cells was employed as a positive control (not shown). In panel B, NK cells from donor D1 were incubated in the presence (solid line) or absence (dotted line) of 50 kU/ml of CA125 for 72 h. Cells were washed with PBS containing 1% BSA, permeabilized with digitonin, and labeled with phycoerythrin (PE) conjugated anti-CD3 ζ (B) antibody. To monitor the expression of ZAP70 (C) and PLC- γ 1 (D), NK cells from D6 and D3, respectively, were incubated in the presence (dotted line) or absence (solid line) of 50 kU/ml of CA125 for 72 h. The cells were permeabilized as discussed in Materials and methods and incubated with unlabeled anti-ZAP70 and anti-PLC- γ 1 antibodies. After washing and labeling with fluorescein isothiocyanate conjugated goat anti-mouse IgG secondary antibody, the cells were analyzed by flow cytometry. All of the flow cytometry studies were performed on NK cells from three donors. Representative experiments are shown.

Effect of CA125 on the expression of CD94/NKG2A

Fc receptor expression is also downregulated in uterine NK cells during pregnancy [27]. CA125 is also induced in the uterus during pregnancy [28,29]. We therefore investigated the effect of CA125 on the expression of CD56 and

CD94/NKG2A using the Z199 antibody that detects this heterodimeric receptor. CD94/NKG2A expression is specifically upregulated on human uterine NK cells during pregnancy [30,31]. Incubation of NK or LAK cells with CA125 did not alter the expression of CD56 (data not shown). On the other hand, CA125 decreased the expression

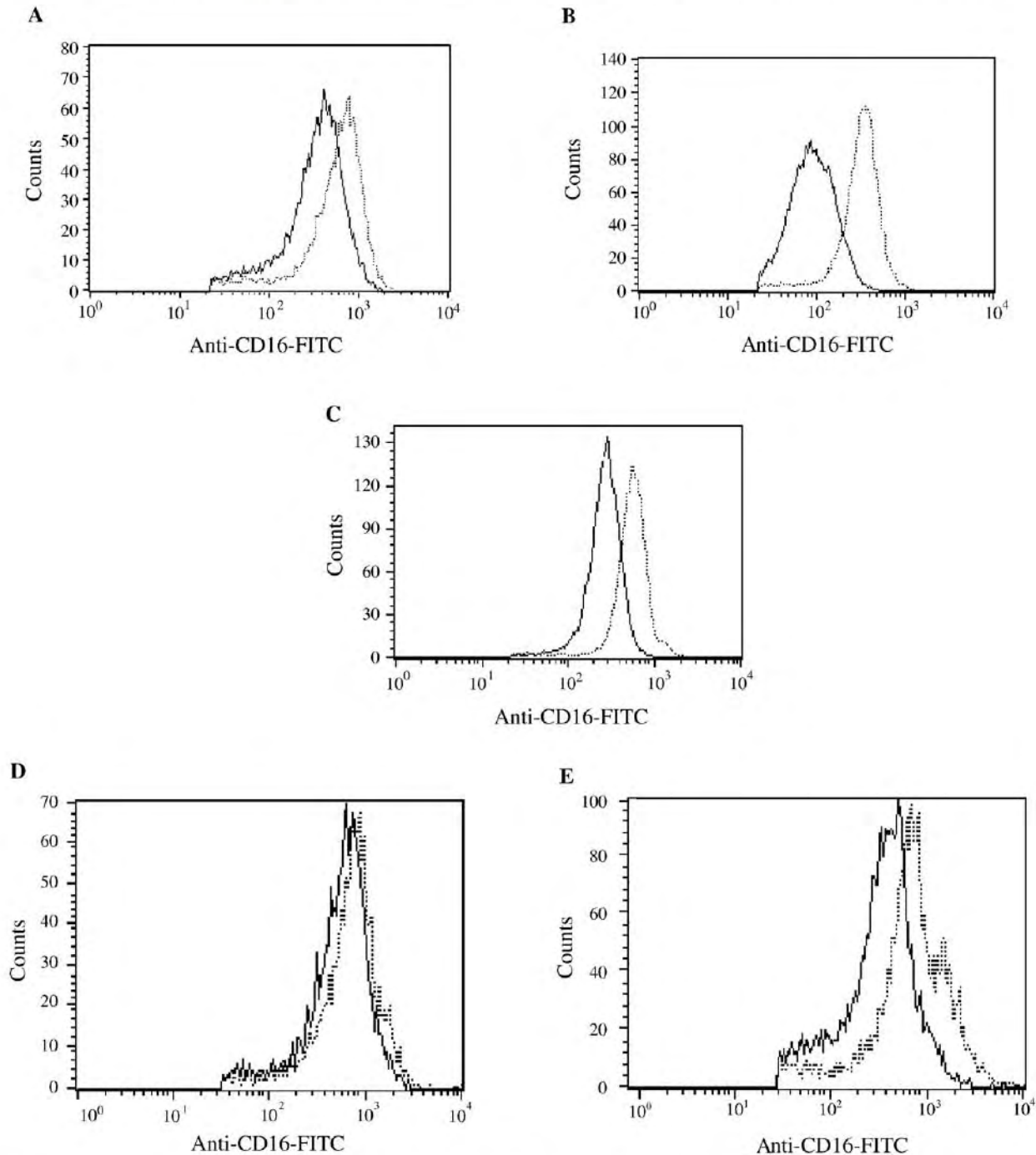


Fig. 6. CA125 downregulates the expression of CD16 on NK cells in a temporally dependent fashion. NK cells from donors D1 (A), D5 (B), and D6 (C) were incubated in the presence (solid line) or absence (dotted line) of 50 kU/ml of CA125 for 72 h. Cells were washed and incubated with fluorescein isothiocyanate-labeled anti-CD16 antibody. Fluorescently tagged cells were analyzed by flow cytometry. The observed loss in MFI was 42% for D1, 70% for D5, and 53% for D6. A time-course analysis of D6 was performed in a separate study. CD16 expression was determined in the absence (dotted line) or presence (solid line) of 50 kU/ml of CA125 for 24 h (D) and 72 h (E). The observed loss in MFI was 18% and 46% after 24- and 72-h incubations, respectively.

Table 1
Effect of CA125 on the expression of CD94/NKG2A

	% CD94/NKG2A positive cells	% Decrease in CD94/NKG2A expression	MFI for CD94/NKG2A positive cells ^b
NK cells from D1			
Control	8.1		769
Test ^a	8.1	0	619
LAK cells from D1			
Control	8.0		1034
Test ^a	7.7	3.8	891
NK cells from D7			
Control	6.3		517
Test ^a	6.8	–	463
LAK cells from D7			
Control	6.3		764
Test ^a	5.8	8.0	639
NK cells from D8			
Control	2.5		453
Test ^a	2.6	–	381
LAK cells from D8			
Control	2.7		776
Test ^a	1.9	30.0	666

^a Test cells were incubated with 50 kU/ml of CA125 for 72 h.

^b P values of test NK and LAK cells versus corresponding controls < 0.05 by one-tailed Student's *t* test.

of CD94/NKG2A on NK and LAK cells by $15.2 \pm 3.0\%$ and $16.3 \pm 1.4\%$, respectively, based on differences in MFI (Table 1).

Discussion

The data obtained in this study demonstrate that CA125 is a potent inhibitor of NK cell-mediated cytotoxicity of tumor cells. This CA125-mediated suppression was observed with NK cells isolated from the peripheral blood of both male and female donors (Fig. 1B). Inhibition of the NK cell response was robust as even the cytotoxic activity of the IL-2-stimulated LAK and the highly active NK-92MI cells was highly attenuated by CA125 (Fig. 3).

The exact mechanism by which CA125 manifests its NK suppressive effect is not clear at this time. One potential mechanism could be that CA125 due to its very large molecular size physically blocks the interaction between the NK and tumor targets. MUC1, another mucin that is also expressed in high amounts on many different tumor cells, has previously been shown to inhibit NK cell function via this mechanism at relatively high concentrations [32].

However, as shown in this study, NK cells had to be incubated for prolonged periods with CA125 before any inhibition of cytotoxicity or downregulation of CD16 expression was observed (Fig. 1). Physical blocking of the effector–target interaction should have resulted in a much more pronounced inhibition of K562 cytotoxicity when the NK cells were preincubated with CA125 for 24 h.

Alternatively, specific interaction of CA125 with cell surface receptors is the likely mechanism employed by this

mucin to suppress the NK cells. In this model, it is postulated that CA125 specifically interacts with either inhibitor or activating receptors on the NK cell surface. CA125 may serve as an antagonist for a specific activating receptor or an agonist for an inhibitory receptor thereby affecting NK cell function. This model is at least partially supported by the data presented in this study.

Prolonged incubation of the NK cells with CA125 leads to significant downregulation of the NK activating receptor CD16. CA125, therefore, induces changes in the NK cells at the molecular level. In a previous study, it was demonstrated that tumor-associated NK cells obtained from patients with EOC show significant reduction in CD16 expression compared to NK cells derived from the peripheral blood of normal human donors [22]. Thus, in our *in vitro* experiments, we have demonstrated that CA125 induces NK cells to acquire a CD16^{dim}/CD56⁺ phenotype that more closely resembles the NK cells present in the epithelial ovarian tumor environment.

Based on the data presented here and our ongoing studies (Hebda and Patankar, unpublished observations), we hypothesize that CA125 specifically binds to a receptor on the NK cell surface and causes major downregulation of activating receptors like CD16. Reduced expression of such activating receptors leads to a relative predominance of the inhibitory receptors on the NK cell surface. Such a shift in receptor expression profile renders the NK cells unable to properly respond and reject the epithelial ovarian tumors. Expression of CA125 should therefore be considered an important strategy employed by these tumors to evade the innate immune response.

Downregulation of CD16 by CA125 likely also helps the ovarian tumors to evade adaptive immune responses. CD16 binds to the Fc portion of the immunoglobulins. Patients with EOC and other cancers possess circulating levels of antibodies against tumor antigens [33]. These antibodies when bound to the cancer cells can activate NK cells via the CD16 receptor. Downregulation of CD16 by CA125 likely results in less efficient binding of the target-bound immunoglobulins by the NK cells thereby compromising the anti-tumor humoral responses. Thus, CA125 may uncouple both antibody-dependent and “natural” killing mechanisms essential for NK cell-mediated lysis.

This immune evasion strategy employed by the ovarian tumors likely can also explain the partial positive benefit accrued in patients responding to anti-CA125 immunotherapy [8,34]. Administration of the anti-CA125 antibodies may not only result in increased recognition of the tumor targets by the immune cells but may also render this mucin incapable of fully manifesting its NK-suppressive effect resulting in increased recognition and killing of the tumors.

Many types of tumor cells including those of ovarian origin downregulate expression of their MHC class I molecules [35]. While loss of MHC class I expression should make ovarian tumor cells less susceptible to MHC class I restricted cytotoxic T lymphocytes, it should greatly

increase their vulnerability to NK cell-mediated lysis [36]. However, loss of MHC class I expression is correlated with very high mortality in many different tumor cell types [37].

In a very elegant study, it was recently demonstrated that EOC patients with increased numbers of cytotoxic T cells show a higher 5-year survival rate [38]. This study implies that the tumor-infiltrating T cells are capable of recognizing and attacking the epithelial ovarian tumors. However, regulatory CD25⁺ T cells (T_{regs}) that inhibit the cytotoxic T cells [39] are also detected within the epithelial ovarian tumor mass. Indeed, the presence of these intratumoral T_{regs} has a negative impact on 5-year survival of patients with EOC [39].

All of these observations on the intratumoral T cells and the data presented in the current study suggest that the epithelial ovarian tumors employ multiple strategies to overcome immune responses. Such strategies likely also include active apoptosis of the tumor-infiltrating immune cells via the Fas–Fas-L pathway as proposed by some investigators [40]. Such redundant and often evolving mechanisms collectively contribute to protection of the cancer cells from immune attack, thereby promoting tumor growth.

While considering the immunosuppressive effect of CA125, it is also important to consider that CA125 is highly expressed by the human decidua [41]. NK cells are the major immune cell type in the decidua and their cytolytic activity is considerably reduced [42]. The decidual NK cells also exhibit decreased expression of CD16 [43]. It is likely that these changes in the decidual NK cells are at least partially induced by CA125.

The developing fetus is antigenically foreign to the mother and should therefore be rejected by the maternal immune system. Because normal development of the fetus is such a vital event, potent immunosuppressive mechanisms are likely employed during pregnancy. We have previously hypothesized that soluble and cell surface-associated glycoconjugates expressed in the decidua play a role in suppressing maternal immune responses against the fetus [44,45]. We would like to propose that the ovarian tumors have co-opted the immune evasion strategies employed by the human fetus to circumvent NK cell responses.

A major impediment in conducting future studies on CA125 is the difficulty in isolating sufficient amounts of purified CA125 from tumor cell lines and from biological sources. We are currently in the process of streamlining a CA125 isolation protocol. Such isolation strategies in conjunction with glycoproteomic analysis and synthesis of recombinant CA125 epitopes will be required for understanding the biology of this mucin.

The use of recently developed murine epithelial ovarian tumor cells will also be of great importance in analyzing the physiological role of CA125 [46,47]. Experiments can be designed to study tumor development using these cell lines in immune-deficient mice. However, the expression of CA125 by these cells must first be clearly demonstrated.

It should also be noted that the structure, post-translational modifications, and gene regulation of murine and human CA125 may differ, thereby complicating the interpretation of the results obtained using these mouse models.

Acknowledgments

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